

Preliminary Two-Dimensional [^1H]NMR Characterization of the Complex Formed between an 18-Amino Acid Peptide Fragment of the α -Subunit of the Nicotinic Acetylcholine Receptor and α -Bungarotoxin^a

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The nicotinic acetylcholine receptor (nAChR) is a ~250 kDa pentameric complex (subunit composition: $\alpha_2\beta\gamma\delta$) which is located at the neuromuscular junction and which is critically involved in eliciting skeletal muscle contraction. The nAChR is a transmembrane protein believed to have a topographical distribution as depicted in FIGURE 1.¹ The snake venom-derived, protein α -neurotoxins, such as α -bungarotoxin (BGTX), which behave as high-affinity, competitive antagonists at the nAChR are believed to bind to an extracellular site at or near the outermost edge or cusp of the receptor (FIG. 1). Although the four subunits are encoded by separate genes, they have a high degree of sequence homology and are presumed to share a common tertiary structure. Each of the subunits is predicted on the basis of primary sequence hydropathy analysis to contain four membrane-spanning regions (M1-M4), with M2 forming the lining of the nonselective cation channel. It is widely held that the first ~200 N-terminal residues from each subunit are extracellular. All four subunits contain a common disulfide loop involving Cys residues 128 and 142 (*Torpedo californica* α -subunit numbering scheme). The α -subunit alone contains an additional, extremely rare vicinal disulfide between residues 192 and 193. Many lines of investigation argue that the major determinants of the agonist and antagonist binding sites are located predominantly on the α -subunit although contributions from the γ - and δ -subunits also appear to be involved. At present, the exact role of the vicinal disulfide in the α -subunit is unclear, but it has been postulated that the conformational movements about the highly strained eight-membered disulfide ring

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may be important in channel activation, subserving in some way the key signal transduction event at the neuromuscular junction.²

Small synthetic peptides corresponding to overlapping regions of the N-terminus (upstream of the M1 region) of the α -subunit have been examined in an attempt to localize the full extent of the α -BGTX binding site.³⁻¹² Several groups now agree that synthetic or recombinant peptide fragments of the α -subunit that contain residues 181-198 (*Torpedo californica*), and which notably include the 192-193 disulfide site within the native receptor, retain significant ability to bind BGTX as evidenced by a variety of binding assays. For example, the 18mer (α 181-YRGWKHWVYYTCCPDTPY-198) binds BGTX with an apparent K_d of 65 nM.^{4,11}

Due to well-documented technical difficulties in preparing crystals of membrane proteins, an atomic resolution structure of the receptor or of its binding site is presently lacking. A 9 Å cryoelectron microscopy derived structure based on

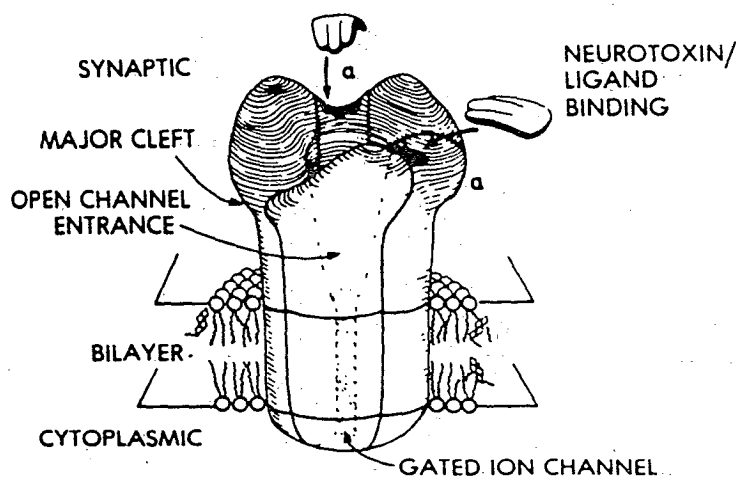


FIGURE 1. Schematic model of the nAChR showing the heteropentameric complex, as viewed in the membrane, as well as the α -bungarotoxin and ligand binding sites. (From Stroud.¹ Reproduced with permission.)

pseudocrystalline arrays of receptor in two-dimensional membranes is providing some moderate resolution insights into the three-dimensional configuration of the receptor in native membranes.¹³ Our interests are in defining the nature of the contact zone between portions of the N-terminal domain of the α -subunit of the nAChR (specifically residues 181-198) and BGTX on an atomic level. We feel that the ability of the 18mer to bind so tightly to the toxin is a good indication that a significantly large fraction (at least 50%) of the actual binding site residues in the native receptor may be included in the 18mer peptide. Hence, we feel that a structural analysis of the receptor peptide fragments bound to BGTX will provide valuable information on the structure, at least that of the antagonist bound state, of the corresponding region in the intact receptor. Towards this end, we have been using two-dimensional [¹H]NMR techniques to characterize the solution structure of the receptor-antagonist complex.

Our current NMR investigations enlarge upon our earlier studies of the disulfide form of a 12-amino acid peptide fragment of the AChR (α 185-196).¹⁴ The view that is emerging from both of our NMR studies is one of a broad contact zone between the proteins. The 12mer has extensive contacts with the first two N-terminal loops of the toxin as evidenced by large chemical shift changes in bound versus free BGTX, as well as by intermolecular NOEs (nuclear Overhauser effects) between the peptide and toxin. The contact zone of the stoichiometric complex formed between BGTX and the 18mer (disulfide form of the peptide) also includes the first two loops of BGTX. The area of contact is extended further, however, on both the N- and C-terminus ends of the nested 12mer sequence (KHWVYYTCCPDT), and we now detect potential contacts with residues in the third loop and an additional residue within the C-terminal tail region.

METHODS

The 12-amino acid peptide corresponding to residues 185-196 (12mer), as well as the 18-amino acid peptide corresponding to residues 181-198 (18mer) of the α -subunit, were synthesized and purified by reverse-phase high-performance liquid chromatography (RP-HPLC) in the Protein and Nucleic Acid Facility, Yale University School of Medicine, New Haven, Connecticut. The primary amino acid sequences are those of the nAChR as isolated from *Torpedo californica* and are highly conserved among species in this region. Both amino and carboxyl termini were prepared unblocked, and the cysteines were prepared and maintained in the reduced state. Susceptibility to covalent alkylation by N-ethylmaleimide, as indicated by a marked increase in retention time on HPLC, confirmed that > 90% of the synthesized peptide contained cysteines in the free sulfhydryl form (see FIG. 2). The amino acid composition of the peptide was verified by ion exchange chromatographic analysis of performic acid oxidation products on a Beckman model 6300 amino acid analyzer. The mass of the 12mer (1,515) and the 18mer (2,340) were confirmed by fast-atom mass bombardment (FAB) techniques, at the Yale Cancer Center Mass Spectroscopy Facility.

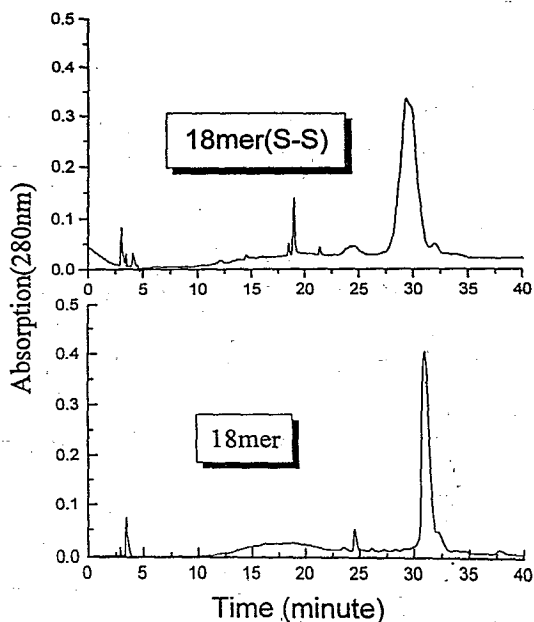
Both the 12mer and the 18mer were oxidized with $K_3Fe(CN)_6$. In both cases, one equivalent of the reduced peptide in CH_3CN/H_2O (10% acetonitrile in the case of the 12mer and 50% acetonitrile for the less soluble 18mer) at 0.13 mM, pH 7.1 was added dropwise with a peristaltic pump to a solution containing 1.2 equivalents of potassium ferricyanide in 0.05 M ammonium bicarbonate at pH 7.1. These conditions, employing slow addition of peptide to oxidant and high dilution were required to prevent intermolecular disulfide formation. Completion of the reaction was monitored with a standard Ellman's reaction. Upon completion of oxidation of the 12mer, 5 mL of Biorad Affigel-501 in ammonium bicarbonate buffer was added to the reaction vessel, and the mixture was gently stirred overnight at 4 °C. After filtration and C18 Sep-Pak desalting, the peptide was purified by C18 RP-HPLC in 50% CH_3CN/H_2O (0.1% TFA). The preparation of the disulfide form of the 18mer did not require the organomercurial step for removal of the residual thiol-containing starting material, as the oxidized and reduced forms of the 18mer were resolvable upon RP-HPLC (see the elution profile in FIG. 3), unlike the oxidized and reduced 12mer, which co-eluted as one broad peak (note the observed retention times, t_R , of the oxidized and reduced 12mer in FIG. 2). The observed broadness of the 12mer-disulfide peak, relative to that of the starting material, suggests that the disulfide form of the peptide may exist as multiple conformers. Consistent with this interpretation is the fact that on the NMR time scale, we have observed, even at elevated

temperatures, multiple low-energy conformers of the 12mer-disulfide peptide suggestive of conformational rearrangements involving the eight-membered disulfide ring (V. J. Basus, unpublished observations). The mass of both disulfide-containing peptides was confirmed by FAB mass spectroscopy, as above. Typical final yields for the 18mer oxidation were 70%, and those for the 12mer were about 50%.

All NMR spectra were acquired on a GE GN-500MHz spectrometer at the University of California at San Francisco. The 1:1 complex of 12mer:BGTX (5 mM each) in H_2O was prepared at pH 5.8, whereas the 1:1 complex of 18mer:BGTX (7.5 mM) was prepared at pH 4.0. In both cases, the peptide in solid form was added to a solution of BGTX.

The ROESY (rotating frame Overhauser spectroscopy) NMR experiment of a 2:1 mixture of BGTX:12mer ($K_d \approx 1.2-3 \mu M$) was carried out at 45 °C, with a 100 ms

FIGURE 2. Reverse-phase (C-18) high-performance liquid chromatography profile of the reduced (bottom) and oxidized (top) 18mer ($\alpha 181-198$). The reduced 18mer has a longer retention time and is baseline separable from the disulfide form of the 18mer.



mixing time, at 7.5 mM BGTX, 3.75 mM peptide. Due to the tighter binding in the complex formed with the 18mer ($K_d \approx 65 \text{ nM}$) the experiment was performed at 65 °C, with a 50 ms mixing time. Usually, the NMR experiments involved an acquired data set of 512×2048 with 16 scans per t_1 increment, and the use of Doddrell's 1:3:3:1 water suppression scheme.

The NOESY (nuclear Overhauser spectroscopy) experiments of the 1:1 peptide:BGTX complex were carried out in temperature intervals of 10 ° beginning from the temperature used for the ROESY experiments of the complex (65 °C for the 18mer complex, 45 °C for the 12mer complex) down to 35 °C where the resonances of free BGTX were assigned. The usual NOESY experiment was run with a mixing time of 150 ms, the above-mentioned data set size, and a "jump and return" water suppression scheme. The HOHAHA (homonuclear Hartman Hahn) experiments were

done with a 50 ms mixing time and a combination of SCUBA, DANTE, and 1:3:3:1 water suppression programs. The DQCOSY (double quantum filtered correlation spectroscopy) spectra were acquired with SCUBA water suppression. All experiments are run in H_2O and the reported chemical shifts are relative to sodium 3-(trimethylsilyl)propionate, or the water resonance.

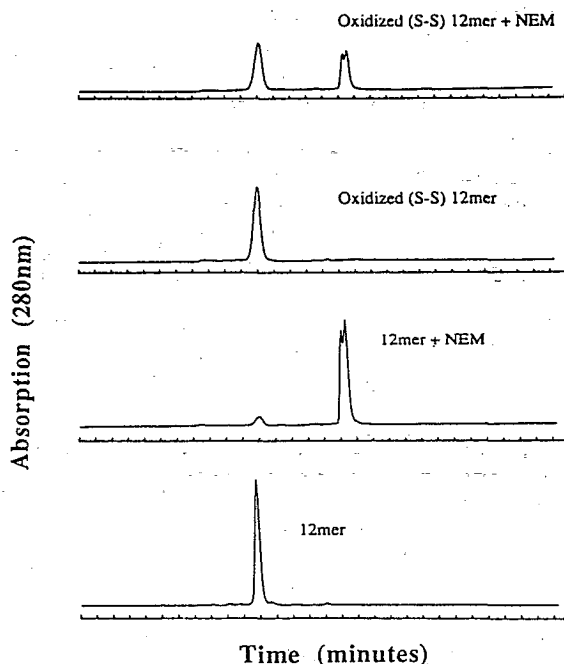


FIGURE 3. Reverse-phase high-performance liquid chromatography (HPLC) analysis of the 12mer ($\alpha 185-196$) before and after ferricyanide oxidation to the disulfide. As the reduced and oxidized form of the 12mer appear to co-migrate upon HPLC, the disulfide form was revealed only after N-ethylmaleimide (NEM) treatment. The NEM alkylates the available sulfhydryl only in the reduced form of the 12mer causing a shift in elution position as indicated. The elution position of the disulfide form of the 12mer is not affected by NEM.

RESULTS AND DISCUSSION

The first step in defining the contact zone between receptor and toxin is the complete 1H assignment of the 1:1 complex (TABLE 1 for 12mer, TABLE 2 for 18mer). To accomplish this, we took advantage of two factors, the difference in sign of chemical exchange cross peaks versus NOE cross peaks in a ROESY experiment of a 2:1 BGTX:18mer complex, and the complete assignment of free BGTX. In the

TABLE 1. ^1H Chemical Shifts^a and Assignments for α -Bungarotoxin Complexed to the AChR Peptide Fragment α 185-196, at 35 °C and pH 5.9

	NH	C α H	C β H	Others
<i>BGTX Residue</i>				
Ile-1	n.o. ^b	4.15	1.53	C γ H ₃ 0.91, C γ H ₂ 1.20, C δ H ₃ 0.74
Val-2	8.11	4.99	1.57	C γ H ₃ 0.57, 0.86
Cys-3	8.76	5.06	2.44, 3.01	
His-4	9.23	5.06	2.89, 2.54	C δ H 6.34, C ϵ H 7.71
Thr-5	9.06	5.29	4.03	C γ H ₃ 1.36
Thr-6	8.62	4.79	5.70	C γ H ₃ 1.27
Ala-7	9.23	4.53	1.42	
Thr-8	7.44	4.68	4.28	C γ H ₃ 1.06
Ser-9	8.28	n.a. ^c	3.79, 3.80	
Pro-10		4.80	2.05, 2.18	
Ile-11	8.85	3.95	1.50	C δ H 0.25, C γ H ₂ 0.91, 1.37
Ser-12	7.66	4.93	3.73, 3.82	
Ala-13	8.19	5.09	0.88	
Val-14	8.90	4.66	2.09	C γ H ₃ 0.89, 0.86
Thr-15	8.47	4.45	4.01	C γ H ₃ 1.24
Cys-16	8.85	4.85	3.35, 3.00	
Pro-17		4.72	2.42, 1.78	
Pro-18		4.31	2.32, 1.88	C δ H ₂ 3.88, 3.60
Gly-19	8.74	4.28, 3.67		
Glu-20	7.91	4.28	2.14, 1.62	
Asn-21	7.82	4.98	3.00, 2.67	N δ H ₂ 6.98, 7.44
Leu-22	8.33	4.99	1.72, 1.54	C γ H 1.58, C δ H ₃ 0.72, 0.79
Cys-23	8.73	5.96	3.27, 2.84	
Tyr-24	9.07	6.00	3.00, 2.66	C δ H 6.65, C ϵ H 6.75
Arg-25	9.00	5.25	n.a.	
Lys-26	9.95	5.81	2.11, 1.88	C γ H 1.60, C ϵ H 2.53
Met-27	9.21	6.24	2.00, 1.98	C γ H ₂ 2.43, 2.62, C ϵ 1.97
Trp-28	8.16	5.20	3.79, 3.45	C η H 7.35, C ξ^3 H 6.80, C ξ^2 H 7.58, C δ H 7.07
Cys-29	9.55	5.40	3.48, 3.06	
Asp-30	8.10 ^d	4.99	3.56, 2.75	
Ala-31	8.30	4.08	1.06	
Phe-32	8.82	4.83	3.10, 2.86	C δ H 7.29, C ξ H 7.35
Cys-33	7.77	4.22	3.76, 3.12	
Ser-34	n.a.	n.a.	n.a.	
Ser-35	n.a.	4.29	n.a.	
Arg-36	8.33	4.57	n.a.	
Gly-37	7.25	4.58, 3.78		
Lys-38	7.81	n.a.	n.a.	
Val-39	8.71	3.79	0.15	C γ H ₃ 0.49, 0.33
Val-40	8.10	4.56	1.48	C γ H ₃ 0.53, 0.47
Glu-41	9.20	5.06	2.40, 2.08	
Leu-42	8.76	5.09	1.55	C δ H 0.89, C γ H 1.52
Gly-43	6.65	4.38, 4.09		
Cys-44	8.40	5.60	3.36, 3.18	
Ala-45	9.34	4.58	1.41	
Ala-46	8.75	4.85	1.54	
Thr-47	7.40	4.33	3.97	C γ H ₃ 1.13
Cys-48	9.00	4.55	n.a.	
Pro-49		4.11	1.52, 2.08	C δ H ₂ 3.57, 3.17, C γ H ₂ 1.0, 1.36
Ser-50	8.02	4.19	3.74, 3.79	
Lys-51	8.23	4.40	n.a.	

(continued)

TABLE 1. (continued)

	NH	C α H	C β H	Others
<i>BGTX Residue (Cont'd)</i>				
Lys-52	8.66	4.49	1.49, 0.79	C γ H ₂ 1.79, 1.32
Pro-53		4.21	1.82, 2.25	
Tyr-54	6.89	4.72	3.53, 3.13	C ϵ H 6.76, C δ H 7.12
Glu-55	7.66	5.11	2.13, 1.92	C γ H 1.81
Glu-56	8.91	4.78	2.10, 2.00	C γ H 2.29
Val-57	8.54	5.30	1.89	C γ H ₃ 0.95, 0.88
Thr-58	9.07	4.77	4.02	C γ H ₃ 1.23
Cys-59	9.13	5.65	3.73	
Cys-60	9.24	5.14	3.61, 3.39	
Ser-61	8.86	4.94	4.20, 3.82	
Thr-62	7.50	4.76	4.29	C γ H ₂ 1.21
Asp-63	8.29	4.82	2.51, 2.31	
Lys-64	9.99	3.17	0.29, 0.98	C δ H 1.52, C γ H ₂ 1.73, 1.49
Cys-65	7.64	4.57	3.77, 3.56	
Asn-66	9.01	4.93	n.a.	N δ H ₂ 7.52, 7.96
Pro-67		3.58	n.a.	
His-68	8.34	3.90	2.82, 2.67	C δ H 6.44, C ϵ H 8.10
Pro-69		n.a.	n.a.	C δ H ₂ 3.13, 2.15, C γ H 1.84
Lys-70	n.a.	4.24	n.a.	
Gln-71	8.10	4.29	2.04	
Arg-72	8.29	4.46	n.a.	
Pro-73		4.48	n.a.	
Gly-74	7.91	3.84		
<i>12mer Residue</i>				
His-186	n.a.	4.74	3.29, 3.09	C δ H 7.78, C ϵ H 8.11
Trp-187	8.79	4.47	3.30, 3.19	C ϵ^2 H 7.28, C δ H 7.01, C ϵ H 7.65, 9.65
Val-188	8.22	4.08	1.96	C γ H ₃ 1.06, 0.68
Tyr-189	7.85	4.85	3.15, 2.79	C δ H 7.31, C ϵ H 6.91
Tyr-190	9.31	5.40	3.33, 2.87	C δ H 7.01, C ϵ H 6.95
Thr-191	n.a.	4.17	4.25	C γ H ₃ 1.19
Cys-192	n.a.	n.a.	n.a.	
Cys-193	7.42	5.34	3.62, 3.38	
Pro-194		n.a.	n.a.	C δ H ₂ 3.94, 3.31
Asp-195	8.20	4.73	2.84, 2.52	
Thr-196	7.73	4.08	4.19	C γ H ₃ 1.12

^aChemical shifts in ppm from internal TSP or water; accuracy +/− 0.01 ppm.^bn.o., not observed.^cn.a., not assigned.^dNote the correction in this shift from that reported erroneously in ref. 14.

ROESY experiment, there was an equimolar amount of "free" and 18mer "bound" BGTX in the sample, and the temperature of the sample (65 °C) was such that there was significant chemical exchange set up between free and bound BGTX. Knowing what the free shifts of BGTX were at the operating temperature (these had to be assigned anew, but were made significantly easier by the assignments that had already been carried out of the toxin at 35 °C and 45 °C) allowed the identification of significantly shifted peaks in the complex. This approach was only useful for resonances that underwent large chemical shift perturbations upon binding, because those nearly similar to those in free toxin were lost in the diagonal noise of the spectrum. Once these "perturbed" residues were assigned, the traditional assign-

TABLE 2. ^1H Chemical Shifts^a and Assignments for α -Bungarotoxin Complexed to the AChR Peptide Fragment α 181-198, at 35 °C and pH 4.0

	NH	C α H	C β H	Others
<i>BGTx Residue</i>				
Ile-1	n.o. ^b	4.17	2.075	C γ H ₃ 0.76, C γ H ₂ 1.16, C δ H ₃ 0.71
Val-2	8.05	4.95	1.52	C γ H ₃ 0.57, 0.84
Cys-3	8.75	5.06	2.39, 2.94	
His-4	9.44	5.05	3.06, 2.95	C δ H 6.42
Thr-5	8.87	5.18	3.99	C γ H ₃ 1.28
Thr-6	8.28	4.43	5.33	C γ H ₃ 1.40
Ala-7	9.19	4.54	1.45	
Thr-8	7.04	4.50	4.28	C γ H ₃ 1.04
Ser-9	8.28	4.54	3.85, 3.56	
Pro-10		n.a. ^c	n.a.	
Ile-11	8.30	4.12	1.68	C γ H ₃ 1.00, C γ H ₂ 1.18, C δ H ₃ 0.88
Ser-12	7.61	4.98	3.95, 3.80	
Ala-13	8.28	5.04	0.83	
Val-14	8.83	4.65	2.05	C γ H ₃ 0.83, 0.86
Thr-15	8.50	4.41	3.98	C γ H ₃ 1.22
Cys-16	8.83	4.84	3.22, 3.00	
Pro-17		n.a.	n.a.	C δ H ₂ 3.49
Pro-18		4.40	2.31, 1.98	
Gly-19	8.77	4.27, 3.66		
Glu-20	7.85	4.28	2.31, 2.19	
Asn-21	8.04	4.94	3.00, 2.65	N δ H ₂ 6.96, 7.44
Leu-22	8.22	4.99	1.69, 1.45	C γ H 1.57, C δ H ₃ 0.69, 0.77
Cys-23	8.75	5.93	3.17, 2.84	
Tyr-24	8.93	5.97	3.00, 2.65	C δ H 6.66
Arg-25	9.03	5.24	n.a.	C δ H ₂ 3.01, NeH 7.16
Lys-26	9.79	5.78	2.15, 1.92	C γ H ₂ 1.60
Met-27	9.18	6.19	2.60, 2.01	C γ H ₂ 1.94, SCH ₃ 1.97
Trp-28	8.19	5.20	3.71, 3.55	C γ H 6.86, C β H 7.28, CeH 7.55, C δ H 7.05, NeH 10.49
Cys-29	9.15	5.10	3.37, 3.03	
Asp-30	9.33	4.98	3.44, 2.73	
Ala-31	8.10	4.04	1.04	
Phe-32	8.80	4.68	3.39, 3.10	C δ H 7.21, C ξ H 7.23
Cys-33	7.67	3.46	3.50, 3.09	
Ser-34	8.94	4.26	4.00, 3.94	
Ser-35	7.83	4.65	3.96	
Arg-36	8.23	4.54	1.87	C γ H ₂ 1.78, 1.65, NeH 7.19
Gly-37	7.19	4.70, 3.72		
Lys-38	8.08	4.20	1.61, 1.48	C γ H 1.25, CeH ₂ 2.97
Val-39	8.65	3.56	0.13	C γ H ₃ 0.38, 0.25
Val-40	8.09	4.48	n.a.	C γ H ₃ 0.46, 0.53
Glu-41	9.20	5.00	2.35	
Leu-42	8.75	5.07	n.a.	C γ H ₂ 1.52, C δ H ₃ 0.92, 0.84
Gly-43	6.62	4.28, 3.98		
Cys-44	8.44	5.53	3.21, 2.99	
Ala-45	9.36	4.58	1.39	
Ala-46	8.73	4.84	1.52	
Thr-47	7.40	4.33	3.95	C γ H ₃ 1.12
Cys-48	9.01	4.54	2.90	
Pro-49		4.15	2.10, 1.54	
Ser-50	8.01	4.13	3.79, 3.74	
Lys-51	8.48	4.24	n.a.	

(continued)

TABLE 2. (continued)

	NH	C α H	C β H	Others
<i>BGTX Residue (Cont'd)</i>				
Lys-52	8.10	4.56	0.96	C γ H ₂ 1.36, 1.31, C δ H ₂ 1.59, 1.47
Pro-53		4.18	2.15, 1.70	
Tyr-54	7.22	4.56	3.16	C ϵ H 6.77
Glu-55	7.73	5.11	2.01	C γ H ₂ 1.90
Glu-56	8.92	4.78	2.37	C γ H ₂ 2.13
Val-57	8.52	5.25	0.87	C γ H ₃ 0.94, 0.85
Thr-58	9.08	4.75	3.99	C γ H ₃ 1.21
Cys-59	9.14	5.62	3.72, 3.02	
Cys-60	9.17	5.12	3.58, 3.41	
Ser-61	8.86	4.92	4.17, 3.79	
Thr-62	7.47	4.75	4.24	C γ H ₃ 1.20
Asp-63	8.37	4.73	2.31	
Lys-64	9.91	3.05	0.96, 0.25	C γ H ₂ 1.52, C δ H ₂ 1.52, 1.49
Cys-65	7.64	4.51	3.74, 3.50	
Asn-66	8.88	4.97	2.54	N δ H ₂ 7.62, 7.53
Pro-67		n.a.	n.a.	
His-68	n.a.	n.a.	n.a.	
Pro-69		n.a.	n.a.	
Lys-70	9.04	4.40	1.92	C ϵ H ₂ 3.00
Gln-71	8.29	4.32	1.97	
Arg-72	8.29	4.63	1.87, 1.75	C γ H ₂ 1.68, 1.46, C δ H ₂ 3.21, N ϵ H 7.16
Pro-73		n.a.	n.a.	
Gly-74	7.96	3.91		
<i>18mer Residue</i>				
Lys-185	7.47	3.11		
Tyr-190	9.35	4.39	2.92	
Thr-191	8.05	4.48	n.a.	
Cys-192	7.69	4.81	n.a.	
Cys-193	7.29	5.42	n.a.	
Pro-194		n.a.	n.a.	C δ H ₂ 4.00, 3.30
Asp-195	8.20	4.90	2.89	
Thr-196	6.97	3.89	4.65	
Tyr-198	9.53	5.36	3.45, 3.06	

^aChemical shifts in ppm from internal TSP or water; accuracy \pm 0.01 ppm.

^bn.o., not observed.

^cn.a., not assigned.

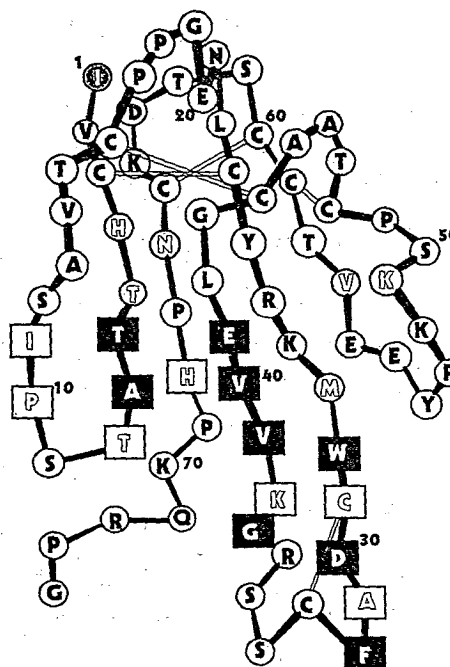
ment protocol of NOESY, HOHAHA, and DQCOSY experiments were performed on the 1:1 complex. It is especially important to note here that we were able to form a true 1:1 complex between BGTX and the peptide fragments. In work recently described using an *Escherichia coli* trpE fusion protein containing an overlapping region of the receptor (α 183–204), < 0.1% of the recombinant protein appeared to be in an active conformation capable of binding toxin.¹⁵

BGTX is a 74-amino acid long, α -neurotoxin from the venom of *Bungarus multicinctus*. BGTX has been extremely useful in pharmacological, electrophysiological, and biochemical studies of the nAChR because of its high selectivity for the receptor and its high binding affinity. Its tertiary structure is characterized by the presence of five disulfide bonds, found between cysteines 3–23, 16–44, 29–33, 48–59, and 60–65. Its high resolution structure has been solved both in solution by

two-dimensional ^1H NMR and in the crystalline state by X-ray crystallography.^{16,17} FIGURE 4 shows that it is a relatively flat, hand-shaped structure composed of 3 loops: the N-terminal loop, the middle so-called toxic loop, and a third loop that leads into the C-terminal tail. The secondary and tertiary structure of BGTX is dominated by a triple-stranded, antiparallel β -sheet including residues Leu22 to Trp28, Val40 to Ala45, and Glu56 to Cys60. The boxed residues in FIGURE 4 are those that undergo large chemical shift perturbations upon binding the 12mer. The circled residues are those that undergo large chemical shift perturbations upon binding the 18mer. BGTX residues that were perturbed both in the binding to the 12mer and to the 18mer receptor fragments are shown as dark filled boxes. The BGTX residues that are most affected by either 12mer or 18mer binding appear to be concentrated in the region of the N-terminal and middle toxic loops.

Residues Asp30 and Arg36 in BGTX have been postulated to form an acetylcholine-mimetic site on the toxin, and might be expected to form the main contact zone with peptide fragments from the receptor.¹⁸ TABLE 3 shows the residues in BGTX that are significantly perturbed ($\Delta\delta > 0.15$ ppm) upon complex formation, and indicates that the contact zone between receptor and toxin is quite extensive. In the solution structure of the 12mer/BGTX complex, as calculated from the interproton distance constraints, the receptor peptide appears to come very close to contributing an additional antiparallel β -strand to the central antiparallel β -sheet structure of the toxin.¹⁴ It is interesting to note from TABLE 3 the number of evolutionarily conserved residues, especially Trp28, Asp30, and Phe32, that are in significantly different

FIGURE 4. Schematic representation of α -bungarotoxin showing its three-looped antiparallel β -sheet structure. The light grey boxed residues are those perturbed significantly ($\Delta\delta > 0.15$ ppm) upon binding the 12mer (α 185–196). The circled residues are those perturbed upon binding the 18mer (α 181–198). Toxin residues perturbed both upon binding the 12mer and the 18mer are shown as dark filled boxes.



chemical environments after BGTX binding to the receptor fragment. The conservation of residues within the α -neurotoxin family of proteins suggests either an important structural or functional role for these conserved residues. The observation that several of the conserved residues are in regions significantly perturbed upon binding the receptor peptide fragments suggests that these residues may indeed play an important functional role in receptor recognition. Although the perturbations of the BGTX side chains may be more informative in terms of revealing direct interactions with the receptor peptide, in addition, a number of large main-chain

TABLE 3. α -Bungarotoxin Residues That Are Significantly Perturbed ($\Delta\delta > 0.15$) upon Binding of the AChR Peptide Fragment α 181-198 at 35 °C and pH 4.0

Residue	Proton	Free α -BGTX (ppm)	Complexed α -BGTX (ppm)	Δ
<u>11</u>	<u>βH</u>	1.87	2.07	0.20
<u>4H</u>	<u>NH</u>	9.18	9.44	0.26
	<u>βH</u>	2.67	3.05	0.38
<u>5T</u>	<u>NH</u>	9.03	8.87	0.16
<u>6T</u>	<u>αH</u>	4.73	4.43	0.30
	<u>βH</u>	5.02	5.32	0.30
7A	α H	4.32	4.54	0.22
<u>27M</u>	<u>αH</u>	6.00	6.19	0.19
<u>28W</u>	<u>NH</u>	8.63	8.19	0.44
30D	NH	8.35	9.33	0.98
	β H	3.17	3.44	0.27
32F	NH	8.27	8.81	0.54
<u>37G</u>	NH	7.71	7.17	0.54
	α H1	4.30	4.70	0.40
	<u>αH2</u>	3.92	3.72	0.20
<u>39V</u>	<u>βH</u>	0.52	0.14	0.38
	<u>γH1</u>	0.48	0.25	0.23
	<u>γH2</u>	0.58	0.39	0.19
<u>40V</u>	NH	8.09	7.70	0.39
	α H	4.78	4.48	0.30
41E	NH	9.38	9.19	0.19
<u>51K</u>	<u>NH</u>	8.30	8.48	0.18
<u>57V</u>	<u>βH</u>	1.87	0.87	1.00
<u>66N</u>	<u>δNH2</u>	7.82	7.62	0.20

NOTE: Residues shown in bold and underlined are those shifted upon binding the 18mer but not the 12mer.

perturbations exist, most especially those involving Asp30 NH. Histograms comparing the shifts of free versus bound BGTX in 12mer complex formation as well as 18mer complex formation of C α H residues are shown in FIGURES 5 and 6, respectively. The extension of the contact zone towards both the N- and C-termini of BGTX is quite easily seen in this analysis, the complexity and diversity of which would be difficult to explain solely on the basis of the 18mer being simply four residues longer on the N-terminus and two residues longer on the C-terminus than the 12mer. The interactions of the 18mer with the third loop are perhaps best

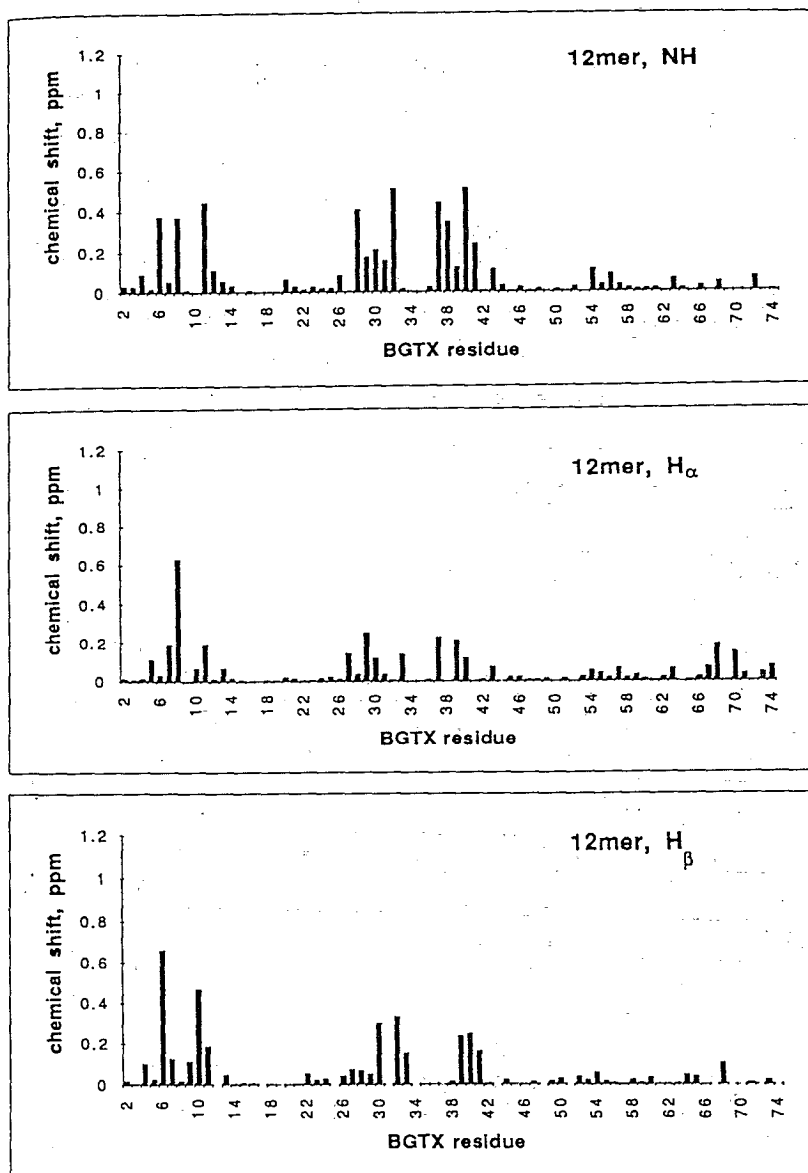


FIGURE 5. Histogram analysis of the chemical shift changes (as absolute values) between α -bungarotoxin "free" and when bound to the 12mer (α 185-196). The *top* profile shows NH shifts, the *middle* one CaH shifts, and the *bottom* one C β H shifts.

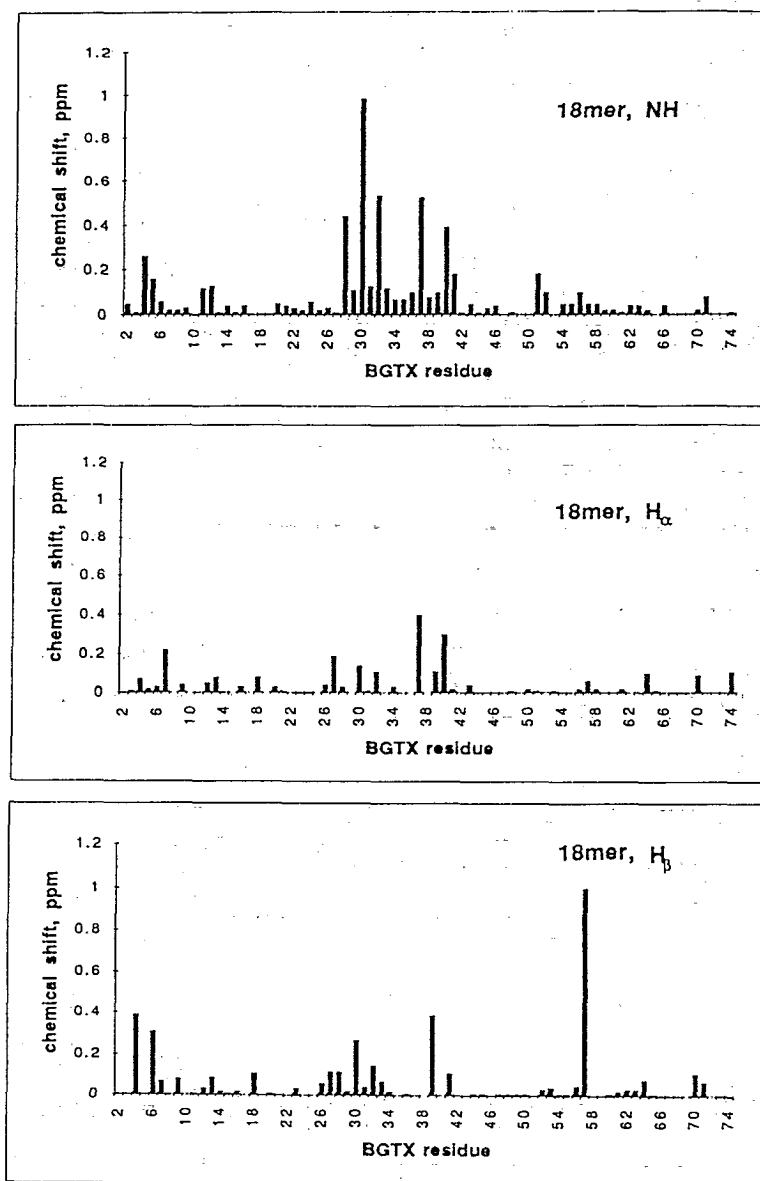


FIGURE 6. Histogram analysis of the chemical shift changes between α -bungarotoxin "free" and when bound to the 18mer (α 181-198). The *top* profile shows NH shifts, the *middle* one $C\alpha H$ shifts, and the *bottom* one $C\beta H$ shifts.

illustrated by the highly significant 1.00 ppm shift of the C β H proton of Val57. Intermolecular NOEs in this region, when available, will be very important in the positioning of the peptide residues near this region, especially the highly conserved Tyr198.

To refine the analysis of the 18mer:BGTX complex, more resonances of the peptide need to be unambiguously assigned. Although most of the main-chain BGTX resonances have been assigned, more than half of the peptide resonances, as seen in TABLE 2, remain obscured by overlap problems related to the large number of proton resonances in a complex of this size. A more complete assignment would allow for the identification of intermolecular NOEs between BGTX and 18mer, generating valuable distance constraints for molecular dynamics and molecular modeling studies based on the experimentally derived parameters. At present, however, the chemical shift overlap in the two dimensions available to us is prohibiting further rapid progress. It is also unfortunate that due to solubility reasons, we are not able to make a 2:1 peptide:toxin complex that might aid us in the assignment of the peptide residues through the use of the ROESY-based strategy that has been so useful in assigning the perturbed BGTX residues. Instead, to address the assignment problem, the chemical synthesis of a universally [^{15}N]labeled synthetic 18mer is in progress. Such a labeled peptide would enable us to use additional powerful heteronuclear NMR experiments to complete the very difficult peptide assignments. Because of the limited supply of [^{15}N]amino acids and the absence of "coupling ready" (fully protected) [^{15}N]amino acids, this approach has been slow. First, complete synthetic protection schemes needed to be worked out for each individual amino acid in the peptide fragment, and then the details of bench-top peptide coupling needed to be explored at the scale determined in part by the cost of the labeled amino acids. The latter is important in this case, as the amount of amino acids needed in commercial peptide synthesizers are unreasonable in light of the cost of the [^{15}N]amino acids. Nevertheless, a [^{15}N]18mer would be extremely useful for [^{15}N]filtered NOESY experiments that could be used to locate peptide residues, as well as to define the intermolecular NOEs between the toxin and the receptor fragment.

To circumvent the problems surrounding the preparation of [^{15}N]labeled synthetic peptides and to provide further insights into the full extent of the contact zone between receptor and BGTX, we designed synthetic genes encoding several longer fragments (46 to 62 residues in length) from within the N-terminal portion of the α -subunit of the nAChR. The synthetic gene constructs are expressed in *E. coli*, and the conditions required for metabolic labeling of the expressed protein with relatively inexpensive [^{15}N]NH $_4$ Cl have also been established.¹⁹ Upon final purification of the labeled recombinant protein fragments, we should be able to perform three-dimensional heteronuclear NMR experiments both in the presence and absence of BGTX. Such studies should greatly enhance our structural knowledge of the receptor in the vicinity of the ligand binding site. If the region to be studied is carefully chosen, a further advantage to working with longer receptor fragments is that longer sequences may encode enough protein folding information to begin to form autonomously folding structural elements or subdomains. It is widely recognized that small peptides (\leq about 25 residues) have little structure of their own, unless they are forming a distinct folding domain (i.e., a zinc finger) of a protein. The longer receptor fragments that we are now exploring should be more likely to fold into structures characteristic of those assumed by these regions in the native receptor. Assuming that the longer fragments remain soluble enough for the NMR investigations, we may therefore be in a good position to compare the structures of the receptor fragment both free in solution and when bound to BGTX or agonists.

We anticipate that the region we have chosen to concentrate our attentions on (α 143–204) will likely form a distinct folding domain, based on the common exon organization in the native gene encoding the α -subunit in all species examined to date. One of the constructs that we are studying contains the 62-amino acid sequence, (α 143–204), joined to the C-terminus region of a bacteriophage coat protein (T7 gene 9 protein). This fusion protein can be overexpressed in *E. coli* without the production of insoluble inclusion bodies. Preliminary BGTX binding studies of this fusion protein show that it binds BGTX with an affinity that is less than twofold reduced from that of intact membranes obtained from the electric organ of *Torpedo californica*. It appears, therefore, that nearly all of the BGTX recognition determinants of the native receptor can be found within this 62-residue region of the α -subunit.

Our NMR-based analysis of the BGTX/receptor peptide fragment contact zone has served to target residues on BGTX for site-directed mutagenesis in order to determine whether the affinity and possibly the specificity for the nAChR could be genetically manipulated. In a genetically engineered system similar to that being used for the overexpression of our receptor fragments, we also constructed a gene for BGTX which allows for the expression of wild-type and site-directed mutants of BGTX.²⁰ We began mutational analysis with an Ala scanning approach, and binding studies of the Asp30Ala mutant toxin have already been completed. Additional mutations involving Arg36Ala and Trp28Ala substitutions are currently being analyzed as well as the Asp30Ala-Arg36Ala double mutation. As the Asp30Ala mutant BGTX appears to bind native nAChR with normal affinity, it appears that the carboxylate side chain of Asp30 is not essential for receptor recognition.²⁰ It is unlikely therefore that the basis for receptor specificity resides in a role for Asp30 as a key component of an acetylcholine mimetic binding site on BGTX. We believe that the NMR structural analysis, together with the functional binding analysis of mutants constructed by site-directed mutagenesis, will continue to be a powerful combination of tools in elucidating the roles of individual residues in both structure and function of the nAChR and of the α -neurotoxin family of proteins.

REFERENCES

1. STROUD, R. M. 1981. Proceedings of the Second SUNYA Conversation in the Discipline of Biomolecular Stereodynamics R. H. Sarma, Ed. 2: 55. Adenine Press. New York.
2. KAO, P. N. & A. KARLIN. 1986. *J. Biol. Chem.* **261**: 8085.
3. WILSON, P. T., T. L. LENTZ & E. HAWROT. 1985. *Proc. Natl. Acad. Sci. USA* **82**: 8790.
4. LENTZ, T. L., E. HAWROT & P. T. WILSON. 1988. *Mol. Pharmacol.* **34**: 643.
5. RADDING, W. P., W. R. CORFIELD, L. S. LEVINSON, G. A. HASSHIM & B. W. LOW. 1988. *FEBS Lett.* **231**: 212.
6. GOTTI, C., G. MAZZOLA, R. LONGHI, D. FORNASARI & F. CLEMENTI. 1987. *Neurosci. Lett.* **228**: 118.
7. RALSTON, S., V. SARIN, H. L. THANH, J. RIVIER, L. FOX & L. J. LINDSTROM. 1987. *Biochemistry* **26**: 3261.
8. NEUMANN, D., D. BARCHAN, M. FRIDKIN & S. FUCHS. 1986. *Proc. Natl. Acad. Sci. USA* **83**: 9250.
9. NEUMANN, D., D. BARCHAN, A. SAFRAN, J. M. GERSHONI & S. FUCHS. 1986. *Proc. Natl. Acad. Sci. USA* **83**: 3008.
10. CONTI-TRONCONI, B. M., F. TANG, B. M. DIETHELM, S. R. SPENCER, S. REINHARDT & A. MAELICKE. 1991. *Biochemistry* **30**: 2575.
11. PEARCE, S. F. A., P. PRESTON-HURLBURT & E. HAWROT. 1990. *Proc. R. Soc. Lond. B* **241**: 207.
12. TZARTOS, S. J. & M. S. REMOUNDOS. 1990. *J. Biol. Chem.* **265**: 21462.

13. UNWIN, N. 1993. *Cell/Neuron* 72/10: 31.
14. BASUS, V. J., G. SONG & E. HAWROT. 1993. *Biochemistry* 32: 12290.
15. FRAENKEL, Y., G. NAVON, A. ARONHEIM & J. M. GERSHONI. 1990. *Biochemistry* 29: 2617.
16. BASUS, V. J., M. BILLETER, R. A. LOVE, R. M. STROUD & I. D. KUNTZ. 1988. *Biochemistry* 27: 2763.
17. LOVE, R. A. & R. M. STROUD. 1986. *Protein Eng.* 1: 37.
18. LOW, B. W., H. S. PRESTON, A. SATO, L. S. ROSEN, T. E. SEARLE, A. D. RUDKO & J. R. RICHARDSON. 1979. *Proc. Natl. Acad. Sci. USA* 73: 2991.
19. GENTILE, L. N., C. A. VASLET, N. J. MESSIER & E. HAWROT. In preparation.
20. ROSENTHAL, J. A., S. H. HSU, N. J. MESSIER, D. SCHNEIDER, C. A. VASLET, L. N. GENTILE & E. HAWROT. 1994. *J. Biol. Chem.* 269: 11178.